# Expression of the Rabies Virus Glycoprotein in Transgenic Tomatoes

**Peter B. McGarvey[1,*], John Hammond[2], Margaret M. Dienelt[2], D. Craig Hooper[3], Zhen Fang Fu[3], Bernhard Dietzschold[3], Hilary Koprowski[1,3] and Frank H. Michaels[1]**

[1]The Biotechnology Foundation Laboratories at Thomas Jefferson University, Department of Microbiology and Immunology, Room M-81 JAH, 1020 Locust St., Philadelphia, PA 19107. [2]Floral and Nursery Plant Research Unit, USDA, ARS, USNA, Room 238, Bldg. 010A, 10300 Baltimore Ave., Beltsville, MD 20705. [3]Center for Neurovirology, Department of Microbiology and Immunology, Thomas Jefferson University, Room 469, JAH, 1020 Locust St., Philadelphia, PA 19107. *Corresponding author (Fax: 215-923-6786, e-mail: pmcgarve@lac.jci.tju.edu).

**We have engineered tomato plants (*Lycopersicon esculentum* Mill var. UC82b) to express a gene for the glycoprotein (G-protein), which coats the outer surface of the rabies virus. The recombinant constructs contained the G-protein gene from the ERA strain of rabies virus, including the signal peptide, under the control of the 35S promoter of cauliflower mosaic virus. Plants were transformed by *Agrobacterium tumefaciens*-mediated transformation of cotyledons and tissue culture on selective media. PCR confirmed the presence of the G-protein gene in plants surviving selection. Northern blot analysis indicated that RNA of the appropriate molecular weight was produced in both leaves and fruit of the transgenic plants. The recombinant G-protein was immunoprecipitated and detected by Western blot from leaves and fruit using different antisera. The G-protein expressed in tomato appeared as two distinct bands with apparent molecular mass of 62 and 60 kDa as compared to the 66 kDa observed for G-protein from virus grown in BHK cells. Electron microscopy of leaf tissue using immunogold-labeling and antisera specific for rabies G-protein showed localization of the G-protein to the Golgi bodies, vesicles, plasmalemma and cell walls of vascular parenchyma cells. In light of our previous demonstration that orally administered rabies G-protein from the same ERA strain elicits protective immunity in animals, these transgenic plants should provide a valuable tool for the development of edible oral vaccines.**

*Received 18 July 1995; accepted 3 October 1995.*

Efforts at mass immunization of humans against rabies in developing countries is hampered by the relatively high cost of vaccine production and by the need for the medical infrastructure required in administering and distributing currently available vaccines. Oral vaccination is considered the ideal approach, and recombinant rabies vaccines ingested orally have proven effective in inducing protective immunity in wildlife against street virus challenge[1,2]. These vaccines are based on the immunogenicity of the well-characterized rabies glycoprotein (G-protein).

As a step toward developing a safe and effective edible oral rabies vaccine, we have explored the use of plants genetically engineered to express viral proteins. Indeed, the hepatitis B surface antigen has been expressed in transgenic tobacco plants[3], binds to antibodies to hepatitis B virus, and is immunogenic in mice[4]. The ideal plant-based oral vaccine would be produced in an edible plant which is consumed fresh

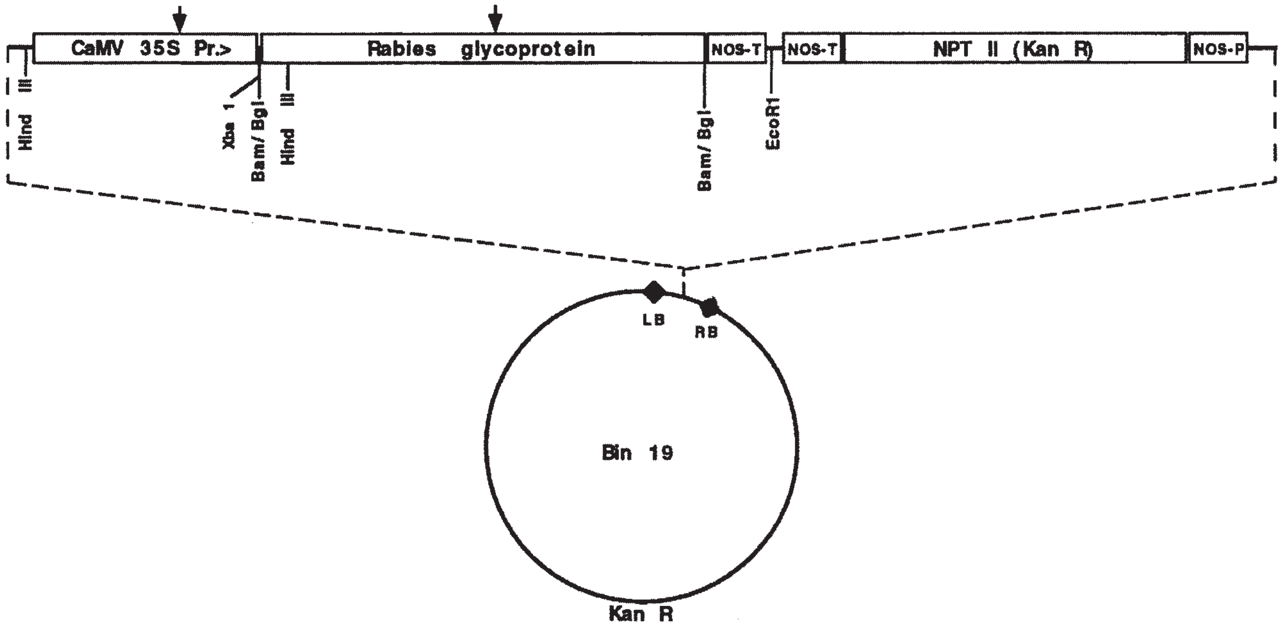


FIGURE 1. The recombinant plasmid pRGRgp containing the rabies G-protein gene under the control of the 35S promoter from cauliflower mosaic virus (CaMV 35S Pr.) and also the nopaline synthase polyadenylation region (NOS-T) constructed from the binary vector RG-2 derived from Bin 19. The neomycin phosphotransferase II (NPTII) gene provides kanamycin resistance in plants and is under the control of the nopaline synthase promoter (NOS-P). Arrows indicate the approximate location of sequences used as PCR primers.

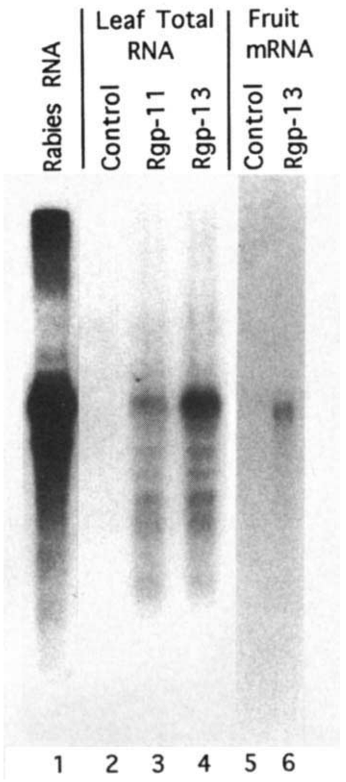


**FIGURE 2.** Northern blot hybridizations of leaf and fruit RNA probed with sequences for the rabies G-protein gene. Lanes: (1) 10 μg of total RNA from rabies-infected BHK cells as standard; (2) 20 μg of total leaf RNA from a normal non-transgenic tomato plant; (3 and 4) 20 μg of total leaf RNA from two primary transgenic tomato plants regenerated from tissue culture and containing the RGRgp construct (Rgp-11 and -13); (5) mRNA from the fruit of a normal tomato plant; and (6) mRNA from the fruit of transgenic tomato plant Rgp-13.

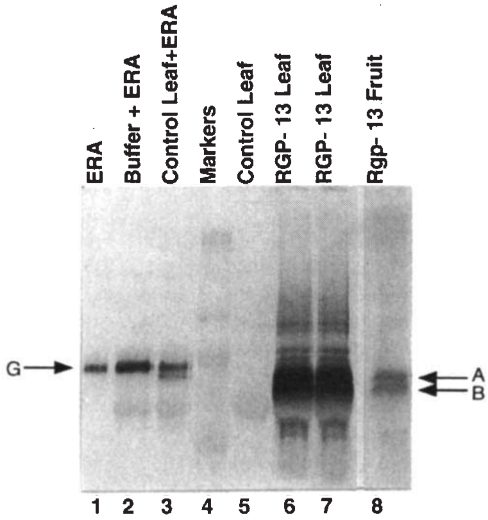


**FIGURE 3.** Western blots of immunoprecipitated protein from transgenic and non-transgenic tomato tissue. Precleared extracts containing 200–500 mg soluble protein were immunoprecipitated with the R165 antiserum (R215 in lane 7) and formalin-fixed *S. aureus* cells. The protein was denatured, electrophoresed through a 7.5% SDS-PAGE gel, and electroblotted onto a PVDF membrane. The Western blot was probed with the mouse monoclonal antibody 6-15C4. Lanes contain protein from: (1) 25 ng inactivated rabies virus; (2) extraction buffer (TSA, 1% Triton, 1% sodium deoxycholic acid) plus 100 ng inactivated rabies virus; (3) control non-transgenic tomato leaves plus 100 ng inactivated rabies virus; (4) prestained molecular weight markers (200, 97.4, 68.0, and 43.0, kDa); (5) control non-transgenic tomato leaf; (6) transgenic tomato leaves precipitated with R215, Rgp-13 line, ($F_1$ generation); (7) same as (6) but precipitated with R165; and (8) transgenic tomato fruit, Rgp-13 line ($F_1$ generation). Band G is the G-protein from purified virus (1 ng virus = 0.33 ng G-protein). Bands A and B are found in transgenic plants expressing the RGRgp transgene. A large very faint band from 50–60 kDa in lanes 2, 3, and 5–8 is from the heavy chain of rabbit IgG.

by the target population.

We report here the construction of transgenic tomatoes expressing the same rabies G-protein gene used in other vaccines and our analysis of its expression and intracellular location in tomato tissues.

## Results

**Plasmid construction.** The construct RGRgp (Fig. 1) was made using a binary *Agrobacterium tumefaciens* vector RG-2 (see Experimental Protocol). RGRgp contains the complete unmodified G-protein gene from the ERA strain of rabies virus under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and also the neomycin phosphotransferase II (NPTII) gene for selection on kanamycin containing medium.

**Plant transformation.** Tomato plants (*Lycopersicon esculentum* Mill var. UC82b) were transformed by *A. tumefaciens*-mediated transformation of cotyledons and tissue culture in selective medium. The plants appeared similar in both morphology and fruiting to non-transgenic tomatoes regenerated from tissue culture. Four lines of transgenic tomatoes containing one or more copies of the RGRgp construct were obtained. Two of these lines expressed G-protein mRNA in leaves and fruit (Fig. 2). One line, designated Rgp-13, was selected for further analysis. Rgp-13 was self-pollinated and the $F_1$ offspring screened for the presence of the recombinant gene by the polymerase chain reaction (PCR). Of 22 $F_1$ progeny, 18 contained the recombinant gene, suggesting that the primary transgenic plant contained a single insert segregating in a 3:1 ratio ($X^2$=0.545, 0.5>$P$<0.2).

**Protein expression.** The recombinant G-protein was purified by immunoprecipitation from extracts of leaf and fruit tissue using two different polyclonal sera, R215 or R165 (see Experimental Protocol), from rabbits immunized with G-protein purified from virus. The G-protein was detected by Western blots (Fig. 3) using a mouse monoclonal antibody specific for a linear virus-neutralizing epitope[5]. The plant-produced G-protein, immunoprecipitated from leaf and fruit tissues, migrated as two prominent bands with apparent molecular mobilities corresponding to 62.5 (±1.6) and 60.0 (±1.0) kDa (Fig. 3, lanes 6, 7, and 8, bands A and B). No bands were precipitated from control tissue (Fig. 3, lane 5). G-protein from denatured rabies virus migrated as a single band at the 66.0 (±1.0) kDa position (Fig. 3, lane 1, band G). When inactivated rabies virus was added to the extraction buffer and immunoprecipitated as a positive control, only a single band was observed (Fig. 3, lane 2). However, when virus was added to extracts of non-transgenic tomato, a second band was observed which appeared to co-migrate with the larger band from transgenic plants (Fig. 3, lane 3). Faint low abundance bands, around 50, 80, and 100 kDa, were observed in some experiments (Fig. 3, lanes 6 and 7) but were absent in others. The amount of G-protein immunoprecipitated was approximately 1–10 ng/mg soluble protein, as estimated by comparison to virus standards on Western blots. Slightly lower amounts were obtained with extracts from fruit.

**Electron microscopy.** The intracellular location of the recombinant rabies G-protein was examined by immunogold labeling of fixed leaf tissue treated with G-protein specific antisera. In $F_1$ generation transgenic tomato plants, gold particles were present in Golgi bodies, vesicles, and cell walls of vascular parenchyma cells (Fig. 4A and B). The highest concentrations of gold were localized in the vesicles and cell walls. Intermittently spaced labeling of the plasmalemma was observed in some sections (Fig. 4A). No labeling of rough endoplasmic reticulum was observed. Cells associated with the leaf vascular system contained more label than other cells. A low to moderate non-specific labeling was seen on chloroplasts and cell walls of control plants (Fig. 4C and D). The gold particle density in transgenic plants was $184.1/\mu m^2$ ($\pm$ 27.3) for Golgi bodies plus vesicles and $102.1/\mu m2$ ($\pm$ 9.7) for cell walls plus plasmalemma compared to $20.8/\mu m^2$ ($\pm$ 14.2) and $14.0/\mu m^2$ ($\pm$ 5.1), respectively, in control plants. Cell walls, Golgi bodies and vesicles were not labeled by an unrelated control antiserum (not shown).

## Discussion

We have engineered transgenic tomato plants to express an unmodified gene for the rabies virus glycoprotein. The protein product of this gene was expressed in leaf and fruit tissue, could be immunoprecipitated by different polyclonal sera to the viral G-protein, and then detected on Western blots using a monoclonal antibody specific for a rabies virus-neutralizing epitope. Immunoprecipitation and detection by different antisera demonstrates that some of the immunologically important epitopes are shared between the G-proteins produced in tomato and BHK cells. The plant-produced G-protein appeared in two major forms with molecular masses about 4 and 6 kDa less than that obtained from virus-infected BHK cells, but still larger than the 58.6 kDa predicted for the unglycosylated polypeptide chain. Since only one mRNA transcript of appropriate size was observed (Fig. 2), the smaller size of the plant G-protein is likely due to the differences between plants and animal cells in the extent of glycosylation and/or processing of N-linked oligosaccharide side chains[6]. The complex glycans of plants are often smaller than those of animals in part due to the absence of sialic acid. The rabies G-protein has 3 sites for N-linked glycosylation of which only two are used in animal cells. Galbraith et al.[7] studied the expression and processing of the surface glycoprotein from another rhabdovirus, vesicular stomatitis virus (VSV), in transiently transfected tobacco protoplasts. Their constructs produced a VSV G-protein which appeared to be correctly processed and localized to the plasma membrane but was about 5 kDa smaller than native VSV G-protein. In addition, they observed a second even smaller form of VSV G-protein, which was produced by a specific proteolysis inhibitable by chymostatin. In our work, mixing purified virus with extracts from normal tomatoes resulted in the appearance of a second band similar in size to the larger G-protein from the transgenic plants (Fig. 3, lane 3), suggesting that a specific enzymatic clevage of amino acid or sugar residues may be responsible for the double bands observerd in both the positive control and transgenic extracts.

Electron microscopy showed localization of the recombinant G-protein to the Golgi bodies, strongly to Golgi-associated vesicles, the plasmalemma, and cell walls of vascular parenchyma cells. Thus, it appears that the recombinant G-protein gene, which was not modified with a plant-derived translation initiation site or signal peptide, contains sufficient information for synthesis and transport similar to that observed in animal cells with other rhabdovirus G-proteins[8].

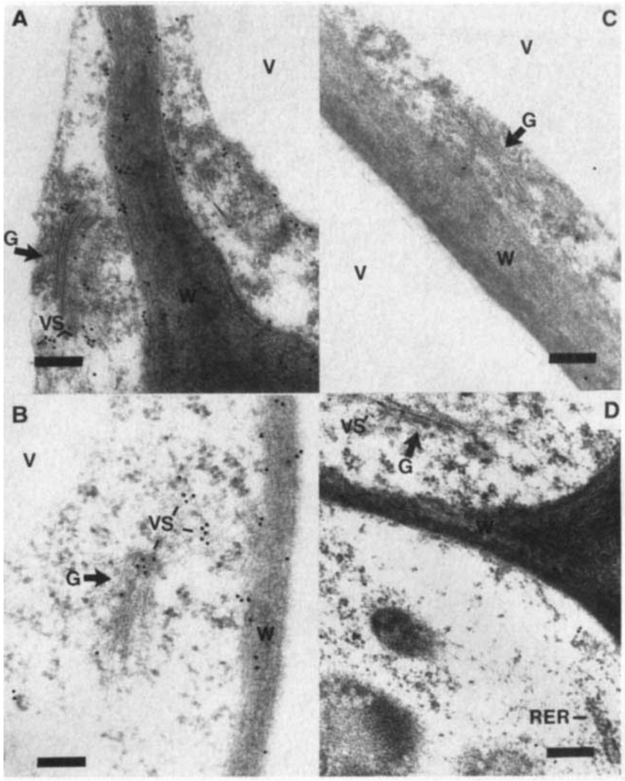


**FIGURE 4. Electron micrographs of vascular parenchyma cells of transgenic and control leaf tissue probed with the rabbit antiserum R165 to the rabies G-protein and labeled with 10 nm gold particles conjugated to goat anti-rabbit IgG. Transgenic plants are the $F_1$ offspring of the self-pollinated plant Rgp-13. Cell structures are labeled: G with arrows point to Golgi bodies; W for cell walls; V for vacuoles; Vs for vesicles, RER for rough endoplasmic reticulum. Panels are: (A) transgenic tomato leaf (plant Rgp-13$F_1$-3) showing gold associated with Golgi bodies (vesicles or edges of cisternae), cell walls, and intermittent regions of the plasmalemma, (bar = 165 nm); (B) transgenic tomato leaf (plant Rgp-13$F_1$-2) showing gold associated similar to panel (A), (bar = 143 nm); (C) non-transgenic control tomato leaf showing sparse labeling of Golgi and cell walls, (bar = 142 nm); (D) non-transgenic control tomato leaf showing low gold background on cell walls, no labeling of Golgi, and no labeling of RER (seen in lower right corner), (bar = 214 nm).**

This work demonstrates the feasibility of expressing the orally immunogenic G-protein from rabies viruses in an edible plant. The expression of the rabies G-protein was low when compared to trangenic plants containing plant- or plant virus-derived genes, but similar to levels seen with equivalent constructs expressing HBsAg in tobacco[3]. Expression of G-protein in plants might be increased by several modifications, including: the use of stronger promoters; the use of plant-derived leader sequences and signal peptides; and targeting the protein for retention in intercellular compartments such as the chloroplasts, vacuole, endoplasmic reticulum, or seed oil-bodies.

Our previous demonstration that recombinant rabies G-protein from baculovirus can immunize raccoons orally[1], coupled with the availability of multiple animal models and knowlege gained from previously developed oral vaccines[2], suggests that rabies virus will provide an excellent model system for studying the interactions of antigens in food and the development of edible oral vaccines in plants. Recently, Haq et al. showed that a highly active oral immunogen, *E. coli* heat-labile enterotoxin, when expressed in transgenic potatoes, can induce a mucosal and systemic antibody response when fed to mice[9]. Studies of the systemic and oral immunogenicity of plant derived G-protein are in progress.

## Experimental Protocol

**Construction of transformation vectors.** The binary *A. tumefaciens* vector RG-2 is a derivative of Bin 19 and similar to pBI121 (ref. 10) except for the orientation of the β-glucuronidase (GUS) gene in relation to the neomycin phosphotransferase II (NPTII) gene. RG-2 was modified using standard procedures[11]. The GUS gene was removed by digestion with SmaI and SstI, treated with T4 DNA polymerase to remove overhanging ends and religated to form RG-G containing a unique BamHI site between the 35S promoter and nopaline synthase (NOS) termination site. A complete cDNA of the ERA rabies virus G-protein was removed from plasmid pTG155 (ref. 12) by digestion with BglII, agarose gel-purified, and inserted into the compatible BamHI site of RG-G to make the plasmid pRGRgp (see Fig. 1), transformed into *E. coli* DH5α, and the orientation of the insert confirmed by restriction mapping.

**Plant transformation.** The pRGRgp plasmid from *E. coli* was transformed into *Agrobacterium tumefaciens* strain LBA4404 by direct transformation[13]. Incorporation of the plasmid was confirmed by restriction digests of DNA from *Agrobacterium* cultures. Tomato tissue (*Lycopersicon esculentum* Mill var. UC82b) was transformed using *Agrobacterium*-mediated plant transformation of cotyledons and a modification of the method of Fillatti et al.[14]. Shoots were generated in medium containing 0.1 mg/ml kanamycin and rooted in medium containing 0.025 mg/ml kanamycin and transferred to soil. The presence of the recombinant gene in regenerated tomato plants was confirmed using PCR[15] with the primers 5′-CACTGACGTAAGGGATGACG-3′ specific for the 35S promoter and 5′-CGTTCACATGAGGATGACACC-3′ specific for the rabies G-protein. PCR posative plants were double checked for incorporation of the recombinant gene by Southern blots[11]. The plants were self-pollinated and the presence of the recombinant construct in the $F_1$ offspring confirmed by PCR.

**Antisera.** Polyclonal antisera R215 and R165 were obtained from rabbits immunized with rabies G-protein purified from virus isolated from BHK cells. Both R215 and R165 immunoprecipitated G-protein from rabies-infected BHK cells (not shown) and from G-protein expressing transgenic plants (Fig. 3, lanes 7 and 8). However, R215, but not R165, binds to denatured G-protein from virus on Western blots indicating that the sera recognize different sets of epitopes on the G-protein. Mouse monoclonal antibody 6-15C4 can neutralize virus infection and recognizes a linear epitope C5-24 from the rabies G-protein[5].

**Analysis of RNA.** Total RNA was purified from 1.0 g of leaf or ripe fruit pericarp tissue using an RNAgents kit (Promega, Madison, WI). Messenger RNA was purified from total RNA using a PolyATract kit (Promega, Madison, WI). RNA (1–20 ug) was denatured with glyoxal, electrophoresed through a 1.5% agarose gel, and blotted to a nylon membrane as described[11]. Northern blots were hybridized with a randomly primed $^{32}$P-labeled plasmid pTG155 containing the rabies G-protein cDNA and visualized by autoradiography.

**Analysis of protein.** Thirty grams of fresh leaf or 50 g of fresh ripe fruit tissue were frozen with liquid nitrogen, ground with mortar and pestle, and homogenized with a Brinkman Polytron in ice-cold TSA buffer (TSA= 0.05 M Tris-HCl pH 8.0, 0.15 M NaCl, 0.025% $NaN_3$) containing 0.5% polyvinylpolypyrrolidone-40, 1.0 mM phenylmethylsulfonylfluoride, 1.0 mM aprotinin and 1 tablet/50 ml of complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). Leaf tissue was homogenized in 3 ml/gram and fruit in 2 ml/gram of buffer. After homogenization one volume of TSA buffer containing 2% Triton X-100 was added and stirred on ice for 1 hr. The mixture was filtered through a layer of Miracloth (Calbiochem, San Diego, CA) and centrifuged at 4,000 x g for 10 min at 4°C. The supernatant was mixed with 0.1 volume of 10% sodium deoxycholic acid on ice for 10 min, and centrifuged at 100,000 x g for 1 hr in a Beckman SW28 rotor. Protein concentration of the supernatant was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) and varied around 12–14 mg/ml for the leaf preparations and 1–2 mg/ml for the fruit preparations. The preparations were stored at −80°C. For immunoprecipitation of plant-produced G-protein 40 ml of plant extract was precleared with 30 μl of normal rabbit sera and 300 μl of a 10% solution of fixed *S. aureus* cells (GibcoBRL, Gaithersburg, MD) as described[11] before addition of 5 μl of polyclonal rabbit serum R215 or R165. The solution was gently mixed on ice for 12–16 h and the IgG fraction precipitated by adding 70 μl of fixed *S. aureus* cells, incubating for 2 h on ice and centrifugation at 4,000 x g for 10 min. The cells were washed once with cold TSA containing 1% Triton X-100 and 1% sodium deoxycholic acid, once with TSA alone, and once with 10 mM Tris-HCl (pH 7.5) 1 mM EDTA. The cell pellet was suspended in 40 μl of 1% SDS loading buffer, electrophoresed through a 7.5% SDS-PAGE gel, and electroblotted onto a PVDF membrane[11]. The membrane was blocked with 5% non-fat dehydrated milk in PBS containing 0.1% Tween-20 overnight, incubated for 1 hr in the same solution containing a 1:1,000 dilution of mouse monoclonal antibody 6-15C4 (ref. 5), and developed using an anti-mouse Vectastain ABC kit (Vector Labs, Burlingame, CA), and 3,3′diaminobenzidine tetrahydrochloride, following the manufacturers directions.

**Electron microscopy.** Three separate $F_1$ generation offspring of the primary transgenic tomato plant Rgp-13 expressing G-protein were sampled. Non-transgenic tomato plants served as controls. Samples no larger than 2 mm² were obtained from partially expanded leaves and fixed in 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M $Na_2HPO_4$-$KH_2PO_4$, pH 7.0, dehydrated in a graded series of ethyl alcohol washes and embedded in LR White (London Resin Co., London, England). Immunogold labeling tests were performed as described[16]. Thin sections on gold grids were blocked for 15 min in 0.01 M Tris-HCL, 0.8 M NaCl, 0.5% Tween-20, 1% BSA (TBS-BSA) and 5% goat normal serum (GNS), rinsed for 5 min in TBS-BSA, and incubated for 90 min in a 1:10 dilution of a rabbit polyclonal antiserum R165 in TBS-BSA, 1% GNS. As a control, additional grids were treated with polyclonal antiserum to impatiens necrotic spot virus HT-1, an unrelated plant virus. Following incubation, grids were rinsed 4 times, 5 min each, in TBS-BSA, blocked for 5 min in TBS-BSA, 5% GNS, and incubated for 60 min in goat anti-rabbit IgG conjugated with 10 nm gold particles (Amersham, Arlington Heights, IL), diluted 1:10 with TBS-BSA, 1% GNS. Grids were then rinsed 4 times, 5 min each, in TBS-BSA and 4 times, 5 min each in double-distilled $H_2O$, and stained with 4% uranyl acetate and Reynolds lead citrate. Samples were examined with a JEOL 100 CX transmission electron microscope. Each labeling experiment was carried out 2 times with 2 grids per serum per plant, and multiple grid openings were examined. Gold particle densities were determined using the software Image and a digital stylus on a Macintosh computer.

## Acknowledgments

We thank George Mossman for technical assistance with electron microscopy. This work was funded in part by the Robert I. Jacobs and William L. Day Funds of the Philadelphia Foundation. Mention of a commercial company or specific equipment does not constitute its endorsement by the authors or the USDA over similar equipment or companies not named.

## References

1. Fu, Z. F., Rupprecht, C. E., Dietzschold, B., Saikumar, P., Niu, H. S., Babka, I., Wunner, W. H. and Koprowski, H. 1993. Oral vaccination of racoons (*Procyon lotor*) with baculovirus-expressed rabies virus glycoprotein. Vaccine **11**:925–928.
2. Rupprecht, C. E., Wiktor, T. J., Johnston, D. H., Hamir, A. N., Dietzschold, B., Wunner, W. H., Glickman, L. T. and Koprowski, H. 1986. Oral immunization and protection of raccoons (*Procyon lotor*) with a vaccinia-rabies glycoprotein recombinant virus vaccine. Proc. Natl Acad. Sci. USA **83**:7949–7950.
3. Mason, H. S., Lam, D. M.-K. and Arntzen, C. J. 1992. Expression of hepatitis B surface antigen in transgenic plants. Proc. Natl. Acad. Sci. USA **89**:11745–11749.
4. Thanavala, Y., Yang, Y.-F., Lyons, P., Mason, H. S. and Arntzen, C. 1995. Immunogenicity of transgenic plant-derived hepatitis B surface antigen. Proc. Natl. Acad. Sci. USA **92**:3358–3361.
5. Dietzschold, B., Gore, M., Marchadier D., Niu, H. S., Bunschoten, H. M., Otvos, L., Wunner, W. H., Ertl, H. C. J., Osterhaus, A. D. M. E. and Koprowski, H. 1990. Structural and immunological characterization of a linear virus neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. J. Virol. **64**:3804–3809.
6. Faye, L., Fitchette-Laine, A.-C., Gomord, V., Chekkafi, A., Delaunay, A.-M. and Driouich, A. 1993. Detection, biosythesis and some functions of glycans N-linked to plant secreted proteins. Society for Experimental Biology Seminar Series **53**:213–242.
7. Galbraith, D. W., Zeiher C. A., Harkins, K. R. and Afonso, C. L. 1992. Biosynthesis, processing and targeting of the G-protein of vesicular stomatitis virus in tobacco protoplasts. Planta **186**:324–336.
8. Doms, R. W., Ruusala, A., Machamer, C., Helenius, J., Helenius, A. and Rose, J. K. 1988. Differential effects of mutations in three domains on folding, quanternary structure, and intracellular transport of Vesicular Stomatitis Virus G protein. J. Cell Biol. **107**:89–99.
9. Haq, T. A., Mason, H. S., Clements, J. D. and Arntzen, C. J. 1995. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science **268**:714–716.
10. Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. 1987. GUS fusions: β-glucuronidase from *Escherichia coli* as a gene fusion marker in higher plants. EMBO J. **6**:3901–3907.
11. Sambrook, J., Fritsch. E. F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory Press, NY.
12. Kieny, M. P., Lathe, R.,Drillien, R., Spehner, D., Skory, S., Schmitt, D., Wiktor, T., Koprowski, H. and Lecocq, J. P. 1984. Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature **312**:163–166.
13. An, G., Ebert, P. R., Nitra, A. and Ha, B. 1988 Binary vectors, p. A3/1–13. *In*: Plant Molecular Biology Manual. Gelvin, S. B. and Schilperoort, R. A. (Eds.). Kluwer Academic, Dordrecht.
14. Fillatti, J. J., Kiser, J., Rose, R. and Comai, L. 1987. Efficient transfer of a glyphosphate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Bio/Technology **5**:726–730.
15. McGarvey, P. B. and Kaper, J. M. 1991. A simple and rapid method for screening transgenic plants using the polymerase chain reaction. BioTechniques **11**:428–432.
16. Lawson, R. H., Dienelt, M. M. and Hsu, H. T. 1993. Effects of passaging a defective isolate of impatiens necrotic spot virus at different temperatures. Phytopathology **83**:662–670.